Conversion of Allyl Alcohol into Acrolein by Rat Liver

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1. Acrolein was detected in rat liver suspensions incubated in the presence of allyl alcohol and allyl formate. Acrolein was determined by condensation of the distilled aldehyde with semicarbazide, followed by spectrophotometric measurement of the semicarbazone at 257 nm and identification by paper chromatography. 2. The transformation of allyl alcohol into acrolein occurred in the presence of NAD^+ . Inhibitors of rat liver alcohol dehydrogenase inhibited the reaction. 3. It is suggested that the hepatotoxic actions of allyl alcohol and of allyl formate on rat liver are related to their conversion into acrolein.

The effect of several hepatotoxic agents depends on their conversion by the liver tissue into more reactive derivatives. Allyl alcohol is toxic for man and mouse (Miessner, 1891), and allyl formate produces hepatonecrosis in rabbits, cats (Piazza, 1915) and rats (Heinemann, 1937). According to a suggestion by Rees & Tarlow (1967), allyl alcohol is metabolized into acrolein by a dehydrogenase present in the post-mitochondrial fraction of liver. In a histochemical study Rees & Tarlow (1967) observed that the alcohol dehydrogenase was mainly localized in the periportal region, as is the necrosis induced by allyl alcohol (Eger, 1954) and by its precursor allyl formate (Popper, 1937). The acrolein produced would cause the damage to cell functions, i.e. decreased protein synthesis and inhibition of respiration, observed during the incubation of rat liver in the presence of allyl alcohol *in vitro*. The compound was not, however, identified.

The present paper deals with the isolation and determination of acrolein in whole homogenates or subcellular fractions of rat liver incubated with allyl alcohol. A method was developed for the determination of small amounts of the aldehyde. Acrolein is separated in the volatile fraction and transformed into its semicarbazone. The concentration of this compound may be determined from its high u.v. extinction.

The results show that postmitochondrial fractions of liver metabolize allyl alcohol to acrolein only in the presence of added NAD^+ . Addition of NAD^+ permits formation of larger amounts of aldehyde in the mitochondria.

Materials and Methods

Chemicals

Uncorrected melting points of the semicarbazones were determined by the sealed-capillary-tube method. The capillary contained sufficient material to ensure

that sublimation did not vitiate the melting-point determination.

Preparation of acrolein and crotonaldehyde semicarbazones

These were prepared as described by Vogel (1962), and were recrystallized from methanol–water (1:1, v/v). The acrolein semicarbazone had m.p. 170°C (literature value m.p. 171°C); light-absorption maximum in water was at 257 nm (ϵ 22300).

The crotonaldehyde semicarbazone had m.p. 197°C (literature value m.p. 199°C); light-absorption maximum in water was at 260 nm (ϵ 26200).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

Animals and preparation of liver fractions

Male albino rats weighing approx. 250 g were used. Livers were homogenized in twice their weight of 0.25 M-sucrose. The homogenates were centrifuged for 10 min at 600g to remove cell debris and nuclei. Mitochondria were separated by centrifugation at 10000g for 10 min and microsomal preparations at 105000g for 1 h. Mitochondrial and microsomal pellets were resuspended in 0.3 M-potassium phosphate buffer, pH 7.4, at 5 mg of protein/ml. Homogenates and postmicrosomal supernatants were diluted with the same buffer at 5 mg of protein/ml.

Incubation and assay

Incubation was performed at 37°C in 25 ml Erlenmeyer flasks. Each flask contained 5 ml of 0.6 M-potassium phosphate buffer, pH 7.4, 0.5 ml of

0.15M-semicarbazide-HCl, 0.4ml of 0.1M-nicotinamide, 0.5ml of tissue suspension and water to a final volume of 10ml. The allyl compounds were added as freshly prepared aqueous solutions. The reaction was stopped with 0.7ml of 70% HClO_4 , the samples were centrifuged and the supernatants transferred to the distillation apparatus described by Cessi & Serafini-Cessi (1963). The first 1ml was collected and the u.v. spectrum (240–280nm) was determined. Then 0.1 ml of 10% (w/v) sodium acetate and 0.1 ml of 0.15M-semicarbazide-HCl were added and after 10min the u.v. spectrum was determined again. The increment at 257nm was calculated.

Isolation and identification by paper chromatography of acrolein from microsomal preparations incubated with allyl alcohol

Bulk incubation was carried out with ten times the amounts of the above-mentioned reagents in a 250ml Erlenmeyer flask. The concentration of allyl alcohol was 4mM and of NAD^+ was $70\mu\text{M}$. Microsomal preparations equivalent to 25mg of protein were added and the reaction was stopped at 40min with 7ml of 70% HClO_4 . The precipitated proteins were removed by centrifugation and the supernatant was distilled in a 250ml distillation flask. The first 1ml was collected and $10\mu\text{l}$ of 0.15M-semicarbazide-HCl added. Under these conditions the yield of semicarbazide calculated from the E_{257} was maximal at 4h. The sample was evaporated to dryness, redissolved with $20\mu\text{l}$ of water and applied to Whatman no. 1 paper. The chromatogram was developed for 12h by the ascending technique with butan-1-ol-acetic acid-water (4:1:5, by vol.). Semi-

carbazones gave yellow spots with *p*-dimethylamino-benzaldehyde reagent on heating at 80°C for 10min.

Results

Determination of acrolein

Acrolein, produced from allyl alcohol added to subcellular fractions of rat liver, was determined as semicarbazone in the first fraction distilled from the incubation mixture. Extinction was a linear function of concentration within the range $0.05\text{--}0.5\mu\text{mol}$. The molar extinction was 1960. The yield of the reaction of the aldehyde with semicarbazide, in the presence of an excess of the latter reagent and sodium acetate, is 50%, calculated from the molar extinction coefficient of semicarbazone. Moreover a purification from interfering non-volatile compounds and concentration of acrolein is achieved by distillation, at the expense of partial recovery. Under the described conditions, 20% of the total acrolein contained in 10ml of the acidified (1M- HClO_4) incubation mixture was found in the first 1ml fraction distilled. The recovery of added acrolein from mixtures containing proteins in concentration similar to that employed in the incubation of cell fractions of liver was about 95% (Table 1) and was considered satisfactory. Recovery became poorer when the concentration of protein and the time of incubation were increased. The specificity of the method was tested by measuring the reactions of other carbonyl compounds involved in cell metabolism with the results recorded in Fig. 1. Simpler aldehydes and ketones do not interfere, as the corresponding carbazones absorb only at shorter wavelengths. Aromatic aldehydes were ruled out

Table 1. *Recovery of acrolein in the presence of microsomal protein*

The conditions of incubation and experimental procedures are given in the Materials and Methods section. The values represent the increment in E_{257} of distilled portions after reaction with semicarbazide. Each sample contained $0.25\mu\text{mol}$ of acrolein.

Time of incubation (min) ... Addition	0*		30	
	ΔE_{257}	%	ΔE_{257}	%
None	0.490	100	0.465	95
Microsomal preparations (2.5mg of protein)	0.500	102	0.460	94
Microsomal preparations (12mg of protein)	0.485	99	0.315	64
Boiled microsomal preparations (2.5mg of protein)	0.475	97	0.460	94
Boiled microsomal preparations (12mg of protein)	0.480	98	0.345	70

* HClO_4 added before acrolein.

because of their strong u.v. extinction. The fractions distilled from the acid supernatants of incubation mixtures, where protein concentration was within the range given above and whether allyl alcohol was added or not, gave negligible extinctions. Crotonaldehyde gave a semicarbazone with a spectrum and a molar extinction comparable with that of acrolein, both derivatives having a strong absorption with $\lambda_{\max}$, at 260 nm owing to the conjugated double bond. The metabolic transformation of allyl alcohol into a C_4 aldehyde seemed unlikely and the possibility was

ruled out by chromatographic identification of the semicarbazone obtained from liver microsomal preparations incubated with allyl alcohol. The R_F values of the two compounds separated by paper chromatography were: acrolein semicarbazone 0.79, crotonaldehyde semicarbazone 0.87. The semicarbazone of the compound obtained from microsomal preparations incubated with allyl alcohol had the same R_F value as an authentic sample of acrolein semicarbazone.

Formation of acrolein by tissue suspensions

The formation of acrolein from allyl alcohol during incubation of homogenates and subcellular fractions occurred to the extent shown in Table 2. The effect of cofactors on conversion into acrolein is also shown in Table 2. No acrolein was detected when NAD^+ was omitted from the incubation mixtures of microsomal preparations and postmicrosomal supernatants. In the incubations with homogenate and mitochondria, small amounts of acrolein were determined and NAD^+ considerably increased the conversion of allyl alcohol into the aldehyde. In the presence of NAD^+ the microsomal fraction had the highest dehydrogenase activity, but $NADP^+$ failed to increase the formation of acrolein. No conversion of allyl alcohol was observed when homogenates or subcellular fractions were incubated by heating at 100°C whether NAD^+ was present or not.

The effect of substrate concentration of the initial velocity of microsomal dehydrogenase activity is shown in Fig. 2 and the effect of incubation time in Fig. 3. From incubation of 2 μ mol of allyl alcohol, 0.1 μ mol of acrolein was detected, representing a 5% conversion of alcohol into aldehyde.

The possibility of transformation into acrolein of other hepatotoxic agents with chemical structures related to allyl alcohol was tested. As shown in Table 3, acrolein was found in the subcellular fractions incubated with allyl formate but not when allylamine was present.

The alcohol dehydrogenase from liver depends on

Fig. 1. U.v. spectra of semicarbazones of acrolein and other carbonyl compounds

Each compound (0.3 μ mol) was dissolved in 10 ml of incubation medium. The tissue suspension was omitted. After acidification with $HClO_4$, the samples were centrifuged to remove insoluble $KClO_4$. Distillation and reaction with semicarbazide was performed as described in the text. Acrolein (—); crotonaldehyde (---); acetone or methyl acetoacetate (-.-.-); formaldehyde or acetaldehyde (.....).

Table 2. Conversion of allyl alcohol by incubation with rat liver suspensions *in vitro*

The experimental conditions and incubation medium are described in the text. Allyl alcohol was added at 2 mM. The results are the mean of two experiments.

Cofactor	Acrolein formed (μ mol/40 min per sample)			
	Homogenates	Mitochondria	Microsomal preparations	Postmicrosomal supernatants
None	0.07	0.08	0.0	0.0
NAD^+ (70 μ M)	0.20	0.18	0.22	0.16
$NADP^+$ (70 μ M)	0.07	0.07	0.0	0.0

free thiol groups for its activity. Kaplan & Ciotti (1953) observed inhibition of liver alcohol dehydrogenase by hydroxylamine. The effect of *p*-chloromercuribenzoate and hydroxylamine on the transformation of alcohol into aldehyde is shown in Table 4. *p*-Chloromercuribenzoate (1mM) completely inhibited the formation of acrolein. The rate of conversion of allyl alcohol was remarkably decreased when microsomal preparations were incubated with hydroxylamine, and the inhibition was proportional to the concentration of the inhibitor.

Discussion

The results of this investigation demonstrate that rat liver *in vitro* converts allyl alcohol and allyl formate into acrolein. This highly reactive aldehyde, by binding to tissue macromolecules, may be respon-

sible for the hepatotoxic action of allyl alcohol and allyl formate.

It has been proposed that the toxic action of several compounds requires metabolic conversion into more reactive intermediates. Butler (1961) suggested that homolytic fission of carbon tetrachloride could occur in liver, producing a free radical with necrogenic effect. Magee & Barnes (1967) have reported that dimethylnitrosamine is metabolized into toxic compounds by liver microsomal enzymes. A similar mechanism has been invoked by Brodie *et al.* (1971) to account for the hepatotoxic activity of bromobenzene. According to these authors the aromatic halogenated hydrocarbons are converted into epoxides that produce necrosis by alkylation of liver proteins. The oxidative microsomal metabolism of these drugs requires NADPH or a regenerating system of reduced nicotinamide cofactors. In contrast, the conversion of allyl alcohol into acrolein, reported

Fig. 2. Effect of concentration of allyl alcohol on its microsomal conversion into acrolein

The incubation time was 20min. NAD⁺ (70 μM) was added to the incubation mixture described in the Materials and Methods section.

Fig. 3. Effect of incubation time on acrolein production by microsomal preparations

The allyl alcohol was at 2mM. The incubation medium was as described in Fig. 2.

Table 3. Formation of acrolein by incubation of subcellular fractions with allyl alcohol and related compounds *in vitro*

The incubation medium was prepared as described in the text, and NAD⁺ (70 μM) was added. The incubation time was 40min. The results are the means of duplicate experiments.

Compound	Acrolein formed (μmol/sample)		
	Mitochondria	Microsomal preparations	Postmicrosomal supernatant
Allyl alcohol (2mM)	0.19	0.23	0.14
Allyl formate (2mM)	0.15	0.21	0.11
Allylamine (2mM)	0.0	0.0	0.0

Table 4. *Effect of alcohol dehydrogenase inhibitors on the conversion of allyl alcohol into acrolein by microsomal preparations*

NAD⁺ (70 μ M) was included in the incubation mixtures described in the text. The concentration of allyl alcohol was 2 mM and the incubation period was 30 min. The results are the mean values of two experiments.

Inhibitor	Inhibition (%)
<i>p</i> -Chloromercuribenzoate (1 mM)	100
Hydroxylamine (10 mM)	66
Hydroxylamine (25 mM)	85

in the present paper, was found to occur in all sub-cellular fractions and was enhanced by the oxidized form of NAD. Arslanian *et al.* (1971) found that rat liver alcohol dehydrogenase is strictly specific for NAD⁺ as cofactor. The observed alcohol-aldehyde conversion does not appear to be a typical drug-metabolizing reaction. The conversion depends on the alcohol dehydrogenase activity of rat liver, and inhibitors of alcohol dehydrogenase abolish the production of acrolein. The extent of allyl alcohol conversion reported in the present paper is not high. This result is consistent with the evidence that allyl alcohol produces necrosis in the periportal region of the liver. It appears that a concentration of toxic aldehyde high enough to induce necrosis may only be obtained in the regions where the alcohol dehydrogenase is present (Rees & Tarlow, 1967). The relation between production of acrolein and localization of necrosis in the periportal region of liver, is also shown by experiments with allyl formate and allylamine. By incubating allyl formate, but not allylamine, acrolein was isolated. Eppinger (1949) observed that both compounds produce liver necrosis, but only allyl formate does this in the periportal zones. This finding is consistent with the work of Reid *et al.* (1970), who reported that the hepatotoxicity of allyl

formate is not affected by previous treatment of the rats with phenobarbital, which activates the drug-metabolizing enzymes.

In conclusion, the evidence reported above shows that allyl alcohol and allyl formate are metabolized by liver into acrolein and that conversion depends on alcohol dehydrogenase activity. The ability of the unsaturated aldehyde to form covalent bonds with tissue proteins in the regions where its production occurs may be responsible for the periportal necrosis induced by allyl alcohol and its precursor allyl formate.

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